ENTEROTOXIN BIOSYNTHESIS BY PROGENY OF REPAIRED HEAT-INJURED CELLS OF *STAPHYLOCOCCUS AUREUS*

J. L. SMITH, M. M. BENCIVENGO and R. L. BUCHANAN

Eastern Reagional Research Center
Agricultural Research Service, U.S. Department of Agriculture
Philadelphia, Pennsylvania 19118

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ABSTRACT

By using a method which permitted the selection of repaired cells from a population of heat-injured and noninjured cells of Staphylococcus aureus *196E, we were able to determine that the progeny of repaired cells retained the ability to produce enterotoxin A (SEA). There were large variations in the amount of SEA produced by the progeny of individual colony forming units (CFU) before and after heating. The average amount of SEA produced by the progeny of noninjured and repaired staphylococci were similar and not significantly different.*

INTRODUCTION

When *Staphylococcus aureus* is subjected to sublethal stress, the cells undergo injury. However, under the right conditions, this damage can be repaired and the cells resume normal cellular activities and growth (Busta 1976; Hurst 1977). Only a limited number of studies have examined the ability of repaired *S. aureus* to resume synthesis of enterotoxin. These studies have indicated that injury does not seem to irreversibly alter the enterotoxin synthesizing capability since heated or freeze-dried injured staphylococci, upon repair, were able to produce enterotoxin B (Collins-Thompson *et al.* 1973; Fung and Vandenbosch 1975). However, those studies employed mixtures of injured and noninjured cells and the observed enterotoxin synthesis could have been restricted to the noninjured cells. The objective of the present study was to better determine if the progeny of repaired *S. aureus* retain the ability to synthesize enterotoxin by employing a system that did not include noninjured cells.

MATERIALS AND METHODS

Preparation of Cells

S. aureus 196E was inoculated into 100 ml tryptic soy broth (Difco) and incubated on a rotary shaker (200 rpm) at 35°C for 16 h. Contents of the culture flasks were centrifuged at 16,000 X g for 5 min at 5°C, washed three times with sterile potassium phosphate buffer (0.1 M, pH 7.2) and resuspended in 3 ml sterile distilled water.

Heat Injury

Flasks containing 50 ml sterile potassium phosphate buffer (0.1 M, pH 7.2) were equilibrated to 49°C (monitored by insertion of a thermocouple below the surface of the buffer). After the flask contents (agitated by magnetic stirring) reached temperature equilibrium, 3 ml washed cells were added; temperature equilibrium was re-established approximately 3 min after addition of the cells. At intervals, aliquots were removed from the flasks and dilutions prepared in 0.1% sterile peptone (Difco) water blanks.

Assay for Injured Cells

Appropriate dilutions were surface plated on tryptic soy agar (TSA; Difco) plus 1% sodium pyruvate (TSAP) and on TSA plus 7% NaCl (TSAS) utilizing a spiral plater (Spiral Systems Instruments, Inc., Bethesda, MD). Plates were counted after 2 days incubation at 35°C. TSAP permits growth of both injured (injured cells must repair before starting to grow) and noninjured cells, while TSAS only allows growth of noninjured cells because heat-injured *S. aureus* lose their salt tolerance (Smolka *et al.* 1974).

Production of Enterotoxin by Repaired Staphylococci

Twenty colonies were picked from TSAP plates (10^{-4} or 10^{-6} dilutions) at each time interval and each colony was transferred to a 50 X 9 petri dish containing 5 ml BHI agar overlaid with a sterile cellophone disk (cellophane-over-BHI agar; Robbins *et al.* 1974). The cellophane prevented diffusion of enterotoxin into the agar medium. The cellophane-over-BHI agar plates were incubated at 37°C for 3 days. Bacterial growth was harvested by adding 1 ml of 0.01 M potassium phosphate buffer (pH 7.2) to the cellophane surface; the cells were removed by centrifugation and the supernatant fluid assayed for enterotoxin.

ELISA Method for Detection of Enterotoxin A (SEA)

The ELISA procedure for determination of SEA was similar to that described by Berdal *et al.* (1981), except that rabbit anti-goat IgG conjugated

with horse-radish peroxidase (Sigma) was used. The enzyme substrate was diammonium 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid). Purified SEA and the monospecific goat and rabbit antisera against SEA were obtained from Dr. Anna Johnson, USAMRIID, Frederick, MD.

RESULTS AND DISCUSSION

The experiment was devised to determine if heat-injured *S. aureus* lost the capacity to produce enterotoxin due to damage of chromosomal or extrachromosomal DNA. Sublethal injury including heat injury has been reported to damage bacterial DNA (Busta 1976; Hurst 1977). The plating procedure permitted separation of colonies that developed from repaired CFU (colony forming units) from colonies that developed from noninjured CFU and, thus, permitted determination of the enterotoxin-producing ability of progeny from repaired CFU in the absence of progeny from noninjured CFU.

The effect of heating *S. aureus* at 49°C on the number of sublethally injured cells (TSAP count minus TSAS count) is presented in Table 1. The number of injured cells increased more than 3 log cycles over the course of heating in experiment A and more than 4 log cycles in experiment B. Some cell death was present also, with TSAP counts being decreased 1 log cycle after 1 and 2 h heating and 2 log cycles after 3 h heating.

The ability of the progeny of repaired *S. aureus* to produce enterotoxin was then evaluated by examining toxin synthesis by isolated colonies from the TSAP plates. The concept underlying this protocol is that each colony on the TSAP plates represents a CFU that was derived either from noninjured or repaired cells. The CFU present on TSAS plates represent noninjured cells only. The probability that any individual colony on a TSAP plate was derived from either a noninjured CFU or repaired CFU will be a function of the relative populations on TSAP and TSAS. For example (Table 1, Exp. B at 1 h), if the count on TSAS was $\sim 3 \times 10^3$ and the count on TSAP was $\sim 7 \times 10^8$; then, only one in $>10^5$ colonies picked off the TSAP plate would represent a colony that developed from a noninjured CFU. The probabilities of picking colonies on TSAP that were derived from noninjured CFU's are presented in Table 1.

The amounts of SEA produced by *S. aureus* derived from two sets of 20 individual colonies isolated from TSAP after 0, 1, 2, and 3 h of heating are presented in Table 2. The colonies labeled A-O and B-O represent unheated samples and are, therefore, the noninjured controls (zero time in Table 1). The data indicate that there are large variations in the amounts of SEA produced by the progeny of individual CFU's before and after heating. Analysis of variance indicated that there was no significant differences between SEA production by colonies isolated in experiments A and B; therefore, the data

Table 1. Injury in *S. aureus* 196E heated in phosphate buffer (0.1 M, pH 7.2) at 49°C

Length of Heating	Sample	Log_{10} *S. aureus*/ml Plated on		Log_{10} Increase in Injured Cells	Probability that a Colony on TSAP was Derived from a Noninjured CFU
		TSAP	TSAS	Δ	
0 h	A	8.89	8.78	0.11	...[a]
	B	8.80	8.92	-0.12	...
1 h	A	8.45	4.15	4.30	<0.0001
	B	8.83	3.43	5.40	<0.00001
2 h	A	8.23	3.36	4.87	<0.0001
	B	8.58	1.43	7.15	<0.0000001
3 h	A	7.08	3.53	3.55	<0.001
	B	7.18	2.56	4.62	<0.0001

[a] Samples were not heated at 0 h.

Table 2. Production of SEA by individual colonies of noninjured and repaired CFU's of *S. aureus* 196E[a]

Concentration of SEA Produced/Colony	No. of Colonies Producing SEA Isolated From							
ng/mg	A-0	B-0	A-1	B-1	A-2	B-2	A-3	B-3
1-100		1	1	1	3	3		2
101-200	2	2	7	2	4	2	2	5
201-300	8	3	9	8	4	3	3	5
301-400	4	9	3	7	5	6	4	3
401-500	5	5		2	2	4	4	4
501-600					2	1	7	1
601-700						1		
701-800								
801-900								
Range (ng/ml)	155-860	121-500	106-370	112-420	67-545	56-700	178-590	100-570
Mean	338		262		299		347	
Standard deviation	129		84		154		147	
Coefficient of variation (%)	38.2		32.1		51.5		42.4	

[a]Injury data is presented in Table 1, A-0 and B-0 are isolates from zero time samples; A-1 and B-1 are isolates from 1 h injury, etc.

was pooled at each time interval to evaluate the effect of repair on toxin production. The average SEA production by progeny of noninjured (A-O, B-O) and repaired (A-1, 2, 3; B-1, 2, 3) CFU's were similar and not significantly different.

The wide variation in SEA production by individual colonies picked from TSAP plates (Table 2) could have been due to differences in the number of cells present on the cellophane-over-BHI plates rather than to differences in

enterotoxin producing capacity of the individual colonies. This potential criticism was tested by determining the SEA-producing capacity of a single colony (from TSAP plate) inoculated onto 10 cellophane-over-BHI plates and comparing to that of 10 colonies inoculated onto individual plates (i.e., a single colony onto a single plate to give 10 plates total). Data from such an experiment are presented in Table 3. The production of enterotoxin from the plates prepared from the single colony fluctuated less than that of the plates from 10 different colonies. Analysis of the data in Table 3 by t-test indicated that there was a significant difference ($p<0.01$) between the means of SEA production from the single colony as compared to that obtained from 10 different colonies. Thus, the variation found in SEA production from the colonies tested in Table 2 is probably due to colonial differences rather than due to differences in cell numbers on the individual cellophane-over-BHI plates.

Table 3. Comparison of SEA production by cells from a single colony inoculated onto 10 cellophane-over-BHI plates versus that of 10 colonies inoculated onto individual plates.

Range of Enterotoxin Production (ng/ml)	Number of Plates Producing SEA	
	From Single Colony	From 10 Colonies
1-100	1	
101-200	7	
201-300		
301-400	2	1
401-500		2
501-600		4
601-700		1
701-800		
801-900		2
Range	93.0-390.4	313.6-850.9
Mean	182.2	537.0

The data presented in Table 2 indicate that sublethal injury does not alter the genetic potential for SEA production by *S. aureus*. The results further indicate that *S. aureus* injured, but not killed, during food processing or cooking operations have the same potential for producing enterotoxin and causing food poisoning as noninjured cells if the injured cells can undergo, repair, and isolation methods must therefore be capable of detecting injured *S. aureus*.

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